NASA TT F-9791

SYNTHESIS OF ADENYLYL-(5' → N)-AMINO ACIDS (PEPTIDES) BY MEANS OF THE CARBODIIMIDE METHOD

T.S.Ryabova, Z.A.Shabarova, and M.A.Prokof'yev

Translation of "Sintez adenilil-(5' → N)-aminokislot (peptidov) karbodiimidnym metodom". Biokhimiya, Vol.30, No.2, pp.235-240, 1965.

NATIONAL AERONAUTICS AND SPACE ADMINISTRATION
WASHINGTON DECEMBER 1965

SYNTHESIS OF ADENYLYL-(5' → N)-AMINO ACIDS (PEPTIDES) BY MEANS OF THE CARBODIIMIDE METHOD

T.S.Ryabova, Z.A.Shabarova, and M.A.Prokof'yev*

19483

Carbodiimide activation of phosphorus acid residue has been used to synthesize adenylyl-(5' → N)-amino acids (peptides). The method developed makes it possible to obtain nucleotide derivatives of esters of amino acids and peptides both for α- and ε-amine groups. Conditions of alkaline hydrolysis of the ester bond in the esters of nucleotidyl-(5' → N)-amino acids are described which provide for a good yield of nucleotidylamino acids with a free carboxyl group. UV and IR spectra, electrophoretic mobility, and R_f values in various systems are given for the nucleotidyl-(5' → N)-amino acids (peptides) synthesized.

Further

To define the role of the nucleotidopeptides, containing a phosphoamide bond (Bibl.1), it is of interest to study the mechanism of conversion in models of such compounds. The formation of a phosphoamide bond between the mononucleotides and the peptide (amino acid) fragments is an important problem in the synthesis of such model structures. In an earlier paper (Bibl.2) we described the use of N,N'-dicyclohexylcarbodiimide (DCC) for this purpose on the example of the synthesis of adenylyl-(5' → N)-phenylalanine ester (Bibl.1).

In this paper, we will describe the application of this reagent to the

---

* Laboratory of Nucleic Acid Chemistry, Faculty of Chemistry, Moscow State University im. M.V.Lomonosov; received April 7, 1964.

** Numbers in the margin indicate pagination in the original foreign text.

synthesis of various amino acid and peptide derivatives of adenylic acid, including its lysine derivative, in which the α-amine group is blocked by the carbobenzoxy group, while the ϵ-amine group participates in the formation of the phosphoamide bond.

1. Method

Systems used in the chromatographic study included: 1) isopropanol, NH_3-H_2O (7:1:2); 2) isoamyl alcohol 5% Na_2HPO_4; 3) butanol, water, CH_3COOH (4:5:1) (supernatant layer). The R_f values given relate to ascending chromatograms. The paper electrophoresis was made in a borate buffer (pH 8.5) and an acetate buffer (pH 4.6) for 5 hrs (6 v/cm).

Leningrad paper "B" was used for both chromatography and electrophoresis. The substances were detected on the chromatograms and electrophoregrams, from the absorption of ultraviolet rays. Compounds with a free amino group were developed by ninhydrin. In determinations of the adenosine-phosphorus-amino acid ratio the quantity of base was calculated from the spectrophotometric data, using the coefficients proposed by Spirin and Belozerskiy (Bibl.3). Total phosphorus was determined by the Barenbloom-Chine method in the Weil-Malherbe and Green modification (Bibl.4). The amino acids (Table 1; I, II, VI) were determined (Bibl.5) in acid hydrolysates (0.1N HCl, 1 hr, 100°C) after chromatography in system 3 [we showed previously (Bibl.6) that, under these conditions, complete hydrolysis of the phosphoamide bond in the compound I takes place]. To determine amino acids in the compound III, the acid hydrolysate was evaporated in vacuo and left standing for 2 hrs at room temperature with glacial acetic acid saturated with HBr (to remove the carbobenzoxy group); the lysine so formed was quantitatively determined after electrophoresis

TABLE 1

YIELD AND PROPERTIES OF ADENYLYL-(5′ → N)-AMINO ACIDS (PEPTIDES)

Compound	Yield,* %	Melting Point, °C	UV Absorption (in Methanol)		R_f in Systems			Electrophoretic Mobility rel. to AMP		Adenosine-Phosphorus-Amino Acid Ratio
			λ_{max}, mμ	E·10⁻³	1	2	3	pH 4.6	pH 8.5	
Methyl ester of adenylyl (5′ → N)-phenylalanine (I)	53	111—112 (decomp.)	258—260	13.0	0.56	0.69	0.30	0.7	0.6	1:0.92:1.09
Methyl ester of adenylyl - (5′ → N)-leucine (NH_4 salt) (II)	62	240 (decomp.)	258—260	12.3	0.59	0.73	0.35	0.7	0.5	1:0.98:0.95
Benzyl ester of adenylyl - (5′ → N^{ε})-N^{ε}-carbobenzoxylysine (III)	40	106—108 (decomp.)	258—260	11.4	0.66	0.48	0.47	0.8	0.6	1:0.83:0.92
Methyl ester of adenylyl - (5′ → N)-alanylvaline (IV)	20	—	259—260	—	0.54	—	—	0.8	0.6	1:0.93:0.75:0.71**
Methyl ester of adenylyl - (5′ → N)-phenylalanylglycine (V)	18	131—133 (decomp.)	258—260	11.0	0.52	0.59	—	0.8	0.7	1:0.78:0.77:1.00**
Adenylyl-(5′ → N)-phenylalanine (VI)	—	220 (decomp.)	258—260	9.5	0.30	0.79	0.30	1	1	1:0.80:0.90
Methyl ester of diadenosinepyrophosphorophenylalanine (VIII)	14	—	258—260	—	0.52	—	—	—	0.7	2:1.7:0.8

* Determined spectrophotometrically after chromatography of the reaction mixture in system 1.

** The ratio of the amino acids entering into the composition of the peptide is given in the order of their appearance after the phosphoric residue.

(6 v/cm, 3 hrs, pH 4.6). In determining the amino acids in the compounds IV and V, hydrolysis was performed with 6N HCl (100° C, 2 hrs).

The methyl esters of leucine, alanylvaline, and phenylalanylglycine and the benzyl ester of α-carbobenzoxylysine were separated from the corresponding salts by treating with chloroform saturated with ammonia; the precipitates were filtered off and the filtrates were carefully evaporated at room temperature to thick oils. Treatment of these esters of the amino acids with an aqueous solution of alkali, followed by extraction with ether, as was done with the methyl ester of phenylalanine, did not give the desired results.

Methyl ester of adenylyl-(5' → N)-leucine(II)*. To 250 mg (0.7 mmol) of AMP was added a solution of freshly prepared methyl ester of leucine (3.5 mmol; 0.5 gm) in 15 ml of dimethylformamide. The reaction mixture was shaken until /237 dissolved; 0.21 ml (1.4 mmol) absolute triethylamine and 280 mg (1.4 mmol) DNA were added, and the mixture was allowed to stand for 3 days in the thermostat at 37° C; the precipitated dicyclohexylurea was filtered off, and the filtrate was precipitated with absolute ether in a centrifuge tube. The oil was triturated several times with absolute ether; the precipitate was decanted, dissolved in 50% methanol, and applied in streaks on four sheets of chromatograph paper. The chromatography was run in system 1. After chromatography, the upper UV-absorbing zone of the chromatogram was eluted with methanol, the eluate was evaporated to dryness in vacuo at room temperature, the residue was triturated with absolute ether, the ether was removed in vacuo, and the substance was dried over P_2O_5 in a vacuum desiccator. Yield of compound II: 90 mg.

Found (m %): C 41.77; H 6.91; N 19.91; $C_{17}H_{27}O_8N_6P \cdot NH_3$.
Calculated (m %): C 41.55; H 6.11; N 19.96.

* The synthesis of the methyl ester of adenylyl-(5' → N)-phenylalanine (I) has been previously described by us (Bibl.2).

Benzyl ester of adenylyl-(5' → N^{ε})-N^{α}-carbobenzoxylysine (III). To 35 mg (0.1 mmol) AMP was added a solution of freshly prepared benzyl ester of α-carbobenzoxylysine (Bibl.7) (185 mg; 0.5 mmol) in 3 ml dimethylformamide. The reaction mixture was shaken until dissolved, after which 0.03 ml (0.2 mmol) of absolute triethylamine and 40 mg DNA (0.2 mmol) were added. Further treatment was as described above. Yield of compound III: 6 mg.

Methyl ester of adenylyl-(5' → N)-alanylvaline (IV). To 250 mg (0.7 mmol) AMP was added a solution of freshly prepared methyl ester of alanylvaline (707 mg; 3.5 mmol) in 15 ml dimethylformamide. The reaction mixture was shaken until dissolved, and 0.21 ml absolute triethylamine (1.4 mmol) and 280 mg (1.4 mmol) DNA were added. Further treatment was as described above. Yield of compound IV: 20 mg.

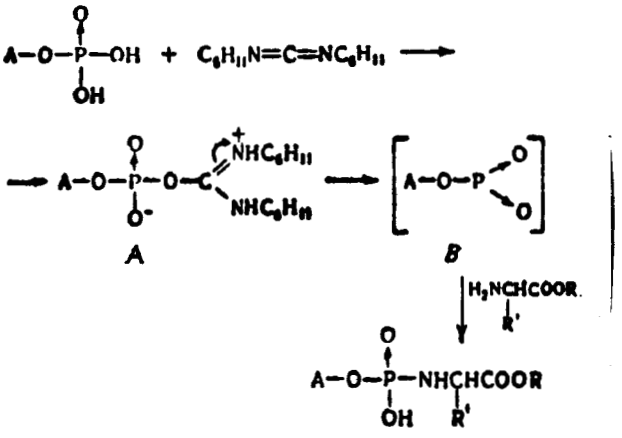
Methyl ester of adenylyl-(5' → N)-phenylalanylglycine (V). To 70 mg (0.2 mmol) AMP was added a solution (235 mg; 1 mmol) of freshly prepared methyl ester of phenylalanylglycine in 5 ml dimethylformamide. The reaction mixture was shaken until dissolved, and 0.06 ml (0.4 mmol) absolute triethylamine and 80 mg (0.4 mmol) DNA were added. The mixture was allowed to stand for 3 days at room temperature. Further treatment was as described above. Yield of compound V: 5 mg.

Adenylyl-(5' → N)-phenylalanine (VI). To 10 mg of compound I was added 0.5 ml of 1N NaOH. The mixture was shaken until dissolved and allowed to stand for 2 hrs in the thermostat at 37°C. To the reaction mixture we added 100 mg of the cationic ion-exchange resin KU-2 (H^+) and stirred for 5 min at room temperature. The resin was filtered off and washed twice with small amounts of water. The filtrate (pH 6) was evaporated to dryness at room temperature, the residue was triturated with absolute ether, the ether was removed in vacuo, and

the substance was dried in a vacuum desiccator over P_2O_5 and paraffin oil. Yield of compound VI: 6 mg.

2. Results and Discussion

To form a phosphoamide bond between adenylic acid and an amino acid or a peptide, we used the widely known method of activating the phosphoric acid residue in its monoesters by means of carbodiimides (Bibl.8). The adduct A



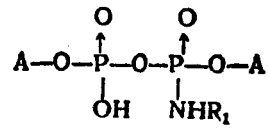
A - residue of adenosine

formed as a result of the reaction of adenylic acid with DNA is, in the proton form, a compound with the highest probability of conversion into the meta- /238 phosphate of the nucleotide (B), which then phosphorylates the ester of the amino acid (peptide), forming the ester of adenylyl-(5' → N)-amino acid (peptide). This reaction, however, is accompanied by side reactions, including the interaction of B with the original nucleotide at the phosphoric acid residue, to form the symmetrical diadenosine pyrophosphate (VII), and also at the hydroxyl group of the sugar, to form the dinucleotide.

To minimize these side reactions, the reaction between AMP and the amino acids (peptides) was made under the following conditions: 1) A five-fold excess

of ester and a two-fold excess of carbodiimide was used, minimizing the formation of compound VII; 2) triethylamine was added to the reaction mixture, which improved the solubility of AMP in dimethylformamide and suppressed the formation of dinucleotide (Bibl.8).

However, it was impossible to eliminate all side reactions that might take place in this reaction. Thus, we obtained considerable quantities of the amino acid (peptide) derivative of diadenosine pyrophosphate* (VIII) whose formation may be represented as a result of the interaction of the ester of adenylyl-(5' → N)-amino acid (peptide) with B. In some cases a considerable amount of



(VIII)

R_1 - residue of amino acid (peptide)

this compound accumulates in the reaction medium. The yield of adenylyl-(5' → N)-amino acids is 50 - 60% (Table 1). The dipeptide derivatives of AMP are formed in a considerably lower yield.

The adenylyl-(5' → N)-amino acids with a free carboxyl group may be obtained by treating the corresponding esters with alkali. In this way, adenylyl-(5' → N)-phenylalanine (VI) was prepared. Table 1 gives a few constants and properties of the resultant compounds. The adenylyl-amino acids (peptides) are hygroscopic and unstable on storage. All of them have the distinct absorption maximum at 258 - 260 mμ which is characteristic of adenine, and give a positive reaction for the cis-glycol grouping and for phosphorus.

In an alkaline buffer (pH 8.5), the compounds I - V, having only one dissociable hydroxyl group, show a considerably lower electrophoretic mobility

---

* Cf. elsewhere (Bibl.2) for the structure of the phenylalanine derivative.

than AMP which latter, at the same pH value, has two hydroxyl groups in the dissociated state. The mobility of the compound VI under these conditions equals the mobility of AMP. In an acid buffer (pH 4.6), the mobility of the compounds I - V approaches that of AMP which was found to have only one hydroxyl group in the dissociated state.

TABLE 2

INFRARED SPECTRA OF COMPOUNDS I - IV AND VI
VIBRATION FREQUENCY (ν), cm^{-1}

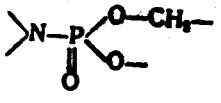
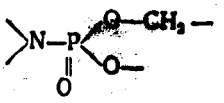
Compound					Assignment of Frequency to Form of Bond
I	II	III	IV	VI	
1736	1734-1732	1739-1929 1714-1712 1702-1695			May be assigned to vibrations of ester carbonyl C = O
1648	1647-1644	1646	1664-1669	1634-1644	Assigned to vibrations of C = C and C = N bonds of the adenine ring
1603	1606-1604	1618	1623-1628	1576-1600	
1576	1576	1574	1572-1578		
1452	1476-1472	1474	1448	1416-1420	Assigned to vibrations of C-O bond of ribose
	1422-1420	1456	1454	1472-1474	
1214	1215	1166-1168	1243	1198-1206	May be assigned to vibrations of P-O bond
	1202	1212-1207			
		1258			
1086	1109	1089-1086	1086-1088	1060-1069	Assigned to vibrations of P-O-C bond
1080	1082	1040	1058-1060		
1072	1068	1128			
822	824-822	825	850	800	Assigned to vibrations of grouping
802	800	800	834		

Table 2 gives the spectral characteristics of the compounds I - IV and VI in the infrared region. A study of the infrared absorption spectra indicated that all of the above compounds show a somewhat similar picture as adenylic acid. The difference lies in the fact that the appearance of absorption bands

in the frequency interval 800 - 850 cm^{-1} is attributable to vibrations of the grouping



For the compounds I - III we found the characteristic frequency of the valence vibrations of the ester carbonyl C = O; in the compound VI, as was to be expected, this frequency was absent. Thus, these spectra are not inconsistent with the structures assigned to the compounds I - IV and VI. One of the methods

TABLE 3

ACID HYDROLYSIS OF COMPOUNDS I - VI

Compound	Hydrolysis Products
I	AMP, adenosine, adenine, phenylalanine, methyl ester of phenylalanine
II	AMP, adenosine, adenine, leucine and its methyl ester
III	AMP, adenosine, adenine, benzyl ester of α-carbobenzoxylysine, α-carbobenzoxylysine, benzyl ester of lysine, lysine
IV	AMP, adenosine, adenine, methyl ester of alanylvaline, alanylvaline, methyl ester of valine, valine, alanine
V	AMP, adenosine, adenine, phenylalanylglycine, phenylalanine, glycine
VI	AMP, adenosine, adenine, phenylalanine

used to demonstrate the structure of the compounds I - VI, was that of hydrolysis under acid and alkaline conditions. The cleavage products found in the acid hydrolysates of these compounds (1N HCl, 10 min, 100° C) after chromatography in system 3, are given in Table 3.

In the alkaline hydrolysates of the compounds I - VI (1N NaOH, 10 min, /240 100° C) we found no substances with a free amino group. Thus, the phosphoamide bond in these compounds is unstable in an acid medium and stable in an alkaline medium. [See also elsewhere (Bibl.1, 5) for the acid lability and alkaline stability of the phosphoamide bond in nucleotidopeptides.]

In acid hydrolysis of adenylyl-(5' → N)-peptides, besides rupture of the phosphoamide bond we also observed a partial rupture of the peptide bond.

3. Conclusions

Carbodiimide activation of the phosphoric acid residue of a nucleotide was utilized to synthesize adenylyl-(5' → N)-amino acids (peptides). This method can be used in preparing nucleotide derivative esters of amino acids and peptides at either the α- or the ϵ-amino group.

We described the conditions of alkaline hydrolysis of the ester bond in esters of nucleotidyl-(5' → N)-amino acids permitting the preparation, at satisfactory yield, of nucleotidylamino acids with a free carboxyl group.

The UV and IR spectra, electrophoretic mobilities, and R_f values in various systems are given for the nucleotidyl-(5' → N)-amino acids synthesized in this manner.

BIBLIOGRAPHY

1. Prokof'yev, M.A., Antonovich, Ye. G., and Bogdanov, A.A.: Biokhimiya, Vol.25, p.931, 1960; Vol.27, pp.266, 442, 1054, 1962.

2. Ribova, T.S., Shabarova, Z.A., and Prokof'yev, M.A.: Dokl. Akad. Nauk SSSR, Vol.153, p.363, 1963.

3. Spirin A.S. and Belozerskiy, A.N.: Biokhimiya, Vol.21, p.768, 1956.

4. Weil-Malherbe, H. and Green, R.: Biochem. J., Vol.49, p.286, 1951.

5. Zaytseva, G.N. and Tyuleneva, N.P.: Lab. delo, Vol.3, p.24, 1958.

6. Savel'yev, Ye.P., Ryabova, T.S., Beletskaya, I.P., and Shabarova, Z.A.: Dokl. Akad. Nauk SSSR, Vol.155, p.1457, 1964.

7. Bezas, B. and Zervas, L.: J. Amer. Chem. Soc., Vol.83, p.719, 1961.

8. Moffatt, J.G. and Khorana, H.G.: J. Amer. Chem. Soc., Vol.83, p.649, 1961.

9. Silayeva, S.A., Kazitsina, L.A., and Prokof'yev, M.A.: Vestn. Mosk. Gos. Univ., No.4, p.75, 1964.

10. Andronova, L.G., Shabarova, Z.A., Ryabova, T.S., and Prokof'yev, M.A.: Zhur. Obshch. Khim., Vol.31, p.3243, 1961.

11. Si-Oh, Li and Eakin, R.: J. Amer. Chem. Soc., Vol.77, p.3519, 1955.